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MECHANISM OF ALGAE-BACTERIA COMPLEXES IN OXIDATION PONDS

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PROGRESS REPORT

MECHANISM OF ALGAE-BACTERIA COMPLEXES IN OXIDATION PONDS

MICROBIOLOGY DEPARTMENT

MISSISSIPPI STATE UNIVERSITY

NOVEMBER 1, 1962

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I.

FACE PAGE

1. Public Health Service Research Grant No. WP-193(C1).

   Mechanism of Algae-Bacteria Complexes in Oxidation Ponds.

2. Principal Investigators.

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   Professor and Head,
   Microbiology Department

   Lewis R. Brown, Ph.D.
   Assistant Professor,
   Microbiology Department

3. Sponsoring institution.

   Mississippi State University
   State College, Mississippi

4. Period covered by the report

   October 1, 1960 to November 1, 1962

5. Date of preparation of the report

   November 1, 1962

II.

SUMMARY STATEMENT

Survey of oxidation ponds in Mississippi has revealed the presence of unusually large numbers of thermophilic aerobic organisms.

Two of these organisms, B. circulans and B. stearothermophilis were grown on both raw and synthetic sewage in a laboratory sewage treatment unit. Preliminary studies in the treatment unit using varied environmental conditions indicated that, under favorable conditions, a considerable reduction in COD values occurred. Moreover, a bioprecipitation reaction was noted which may be of considerable value in the removal of sewage solids.

Further studies proved that B. circulans apparently required carbon dioxide for spore germination. In addition, it was found that the carbon dioxide phenomenon is related inversely to the occurrence of bioprecipitation.

Extensive studies of inorganic cations and anions including nitrate, nitrite, chloride, phosphate, sodium, potassium and iron proved that no large effect, either positive or negative, was evident.

Tests for reductions in coliform population by the thermophilic aerobes used indicate almost complete elimination (99.9%) of the true coliforms.

III.

FULL STATEMENT OF PROGRESS

INTRODUCTION

The title of the present research project is Mechanism of Algae-Bacteria Complexes in Oxidation Ponds. During the course of this investigation it was found that practically all of the ponds tested contained some thermophilic microorganisms. Studies conducted with these thermophiles indicated that they held considerable potential as a means of stabilizing domestic sewage. Therefore, the title "Mechanisms of Sewage Stabilization by Thermophiles" has been substituted in the renewal proposal.

It seemed desirable to include in this progress report only that material relating to sewage stabilization by aerobic thermophiles. For ease of presentation the report is divided into four sections, the first three of which have been accepted for publication. The fourth section deals with some preliminary finding on the fate of coliform organisms during the aerobic thermophilic process.

1. THE DECOMPOSITION OF SEWAGE BY THERMOPHILIC MICROORGANISMS*

I. Preliminary Studies

by Robert G. Tischer, Lewis R. Brown and David W. Cook.

Mississippi State University, State College, Mississippi

From a survey of the microbiological population of oxidation ponds in Mississippi, it was found that many of the ponds contained some thermophilic microorganisms. In fact, in some cases the concentration of these organisms seemed disproportionally high. For example, one of the ponds had between 10^4 and 10^5 organisms capable of growing at 55°C, between 10^7 and 10^8 organisms capable of growing at 37°C and between 10^6 and 10^7 organisms capable of growing at 20°C. Generally, however, the concentration of organisms capable of growing at 55°C was between 1 and 100 organisms per ml. Representative thermophiles were isolated from each of the samples and further tests showed that the majority of them were obligate thermophiles (i.e., no growth below 45°C).

The question arises as to whether these thermophiles have merely been introduced into the ponds and survived or whether they are actively increasing in numbers. Not irrelevent to this question is the matter of whether these organisms play any significant part in the purification of the sewage. Also of interest is the question as to whether a treatment facility operated at thermophilic temperatures would be of some practical value in treating sewage.

This first paper will report on some of the preliminary findings on the decomposition of sewage by a thermophilic bacterium.

*This investigation was supported in part by a Public Health Service Research Grant No. WP-193(C1) from the Division of Water Supply and Pollution Control, National Institutes of Health, Public Health Service.

PROCEDURES

The organism used in this study was isolated from a sewage lagoon located at Waynesboro, Mississippi by the conventional enrichment technique using yeast extract peptone broth and an incubation temperature of 55°C. A pure culture of this organism was obtained by streaking from the liquid enrichment onto the same medium solidified by the addition of 1.5% Bacto-Agar.

All differential tests were conducted according to the Manual of Microbiological Methods, (1957).

The Bacto-leucine assay medium employed as synthetic sewage was modified by the addition of 0.16 g leucine, 0.005 g sodium lauryl sulfate and 0.005 g stearic acid per liter. Normally 1 liter of this medium was diluted to 20 liters with deionized water.

The domestic sewage was obtained from the Starkville, Mississippi sewage system after preliminary screening. It was sterilized by autoclaving at 121°C for 15 minutes.

Cencentrated domestic sewage was prepared by boiling raw domestic sewage under a vacuum until the volume had been reduced approximately 75%.

The dichromate reflux method was employed for determining the Chemical Oxygen Demand (COD) according to the method described in the Standard Methods for the Examination of Water, Sewage, and Industrial Wastes (1955).

RESULTS

Classification of the bacterium. This organism is a motile gram positive spore forming rod. The vegetative cells are 3.2 μ x 0.69 μ and the spores are 1.64 μ x 1.15 μ. The spores are subterminal and the sporangium is bulged. No acid was produced in either glucose or

lactose medium, gelatin was not liquified. Starch was hydrolized, nitrates were not reduced to nitrite and acetylmethylcarbinol was not produced. Litmus milk was unchanged except for an alkaline curd.

In modified Bacto-leucine assay broth this organism showed optimum growth at 55°C and slight growth at 45°C. No growth was observed at 37°C or 65°C.

This organism has been tentatively classified as Bacillus circulans. (Breed, Murray and Smith, 1957).

Growth experiments using synthetic artificial sewage.

This organism was tested for its ability to reduce the COD of the synthetic sewage. For this experiment liter quantities of this medium were dispensed in 2 liter culture flasks, inoculated and incubated at 55°C under stationary conditions. The results of this experiment are shown in Table I. It was observed that the growth was not especially heavy and, as can be seen, very little reduction in COD of the medium took place. This seemed to indicate either a lack of utilizable substrate or a lack of dissolved oxygen. Therefore, the following experiment was conducted. The strength of the synthetic sewage was doubled and dispensed in one hundred ml. quantities in 12 oz. prescription bottles and inoculated. Half of the bottles were placed on a reciprocating shaker (approximately 96 strokes per minute). Incubation was at 55°C. The results of this experiment are shown in Table II. From these results it seems reasonable to assume that a lack of a sufficient quantity of dissolved oxygen was limiting the growth of this organism in the synthetic sewage. This conclusion is further substantiated by comparing the data from this experiment conducted under stationary conditions with that obtained in the preceding experiment. In this experiment the concentration of substrate was increased while the

surface area of the liquid was decreased. As can be observed, better COD reduction took place in the former experiment than in this experiment using stationary incubation.

Growth experiments using domestic sewage.

A test was conducted to determine the ability of this organism to reduce the COD of domestic sewage under varying conditions of aeration. One hundred ml. amounts of sterile sewage were dispensed into 12 oz. prescription bottles, inoculated, and incubated at 55°C either under stationary or shake conditions. Additionally 1 liter of this sewage was placed in a sterile glass column (2-inch diameter x 4 feet in length) and aerated using compressed air. The results of this experiment are shown in Table III. It is clear that the amount of aeration provided by the column technique materially increased the oxidation of the sewage.

Concentrated domestic sewage was placed in the aerated column, inoculated and sampled periodically. The results are shown in Table IV. Repeated trials using domestic sewage, both normal and concentrated, agreed substantially with the results shown above. For these experiments, the size and age of the inoculum varied as well as the source. Sometimes the inoculum was grown in nutrient broth while at other times it was grown in the synthetic sewage or domestic sewage.

In order to be sure that the reduction in COD in the above experiments using the aerated sewage was due to the action of B. circulans and not aeration alone, the following experiment was conducted. One liter of sterile sewage was placed in the column and aerated for 41 hours. The results as shown in Table V proved that aeration alone did not reduce the COD of the sewage.

On occasion it was observed that the sewage in the column cleared

rather than becoming turbid with an apparent increase in precipitate. Since the COD analyses were made on the whole sewage after incubation without allowing any settling to take place, this observation led to the interesting possibility that this organism was causing a precipitation of some of the material in the sewage with the accompanying and subsequent utilization of the precipitated material. Consequently, sterile sewage was placed in the column, inoculated and aerated at 55° C. Samples were collected at 0, 18, and 43 hours and analyzed. Also, a portion of each of these samples were subjected to centrifugation and the supernatant analyzed. The results are shown in Table VI. Although the analysis of the whole sewage after 18 hours showed only a 15 per cent reduction in COD, the reduction in COD of the supernatant material was 47 per cent. After 43 hours, however, the percent reduction in the whole sample had increased while the percent reduction in the supernatant after centrifugation had decreased, indicating that a portion of the material precipitated out after 18 hours had been solubilized by the organisms and gone into solution. The differences in response between the COD reductions in this experiment and the former ones were attributed to changes in the amount of inoculum and in the composition of the sewage itself. In this experiment a small inoculum was used (less than 0.5%) whereas in the earlier ones a 10% inoculum was used.

Further evidence of this bio-precipitation reaction was observed, in shake cultures of this organism using domestic sewage as the substrate. Additional tests were conducted using filtered domestic sewage containing 0.5% Bacto-peptone and 0.3% Bacto-beef extract. The growth of B. circulans in this medium was exemplified by an increase in turbidity as well as the formation of a heavy brownish granular

precipitate. With continued incubation, the quantity of precipitate decreased although it did not disappear completely. In all probability, the build-up of waste products served to terminate growth before all of the precipitate was digested. It should be pointed out that the precipitation reaction was never observed under any conditions when the synthetic sewage was employed as the substrate.

DISCUSSION

In this preliminary study only the COD analysis was employed and consequently no evaluation of the effectiveness of a thermophilic system for treating sewage can be made at this point since the principle measure of performance of a sewage treatment process is its efficiency in reducing the Biochemical Oxygen Demand (BOD) and the suspended solids content of the influent. Tests of BOD values will be reported in a later communication. The results obtained thus far, however, using a thermophilic bacterium to decompose domestic sewage are sufficiently encouraging to warrant further study.

With the organism employed herein it was shown that aeration greatly increased the decomposition of the substrate and suggests that column aeration is more efficient than the shaking methods employed.

Of particular significance was the observation that the test organism caused a bio-precipitation of the sewage. The results suggest that the material precipitated is at least in part protein in nature. This factor alone may well be of practical significance in treating sewage and will be reported in a later publication.

At the present time very little thought has been given to the possibility of designing a thermophilic sewage treatment plant. A number of advantages and disadvantages are readily apparant. Probably the most obvious disadvantage is the increased cost of operation. This

problem, however, may be solved by the use of a cheap energy source such as a solar furnace or atomic energy. On the other hand, the use of a thermophilic system should provide for a greater control of the biological population since fewer organisms are capable of thriving under these conditions. Also such a system should eliminate the necessity of chlorinating the effluent for the sewage in effect would be pasteurized.

ACKNOWLEDGEMENTS

The continued assistance and cooperation of the Mississippi State Board of Health and members of their field staff in obtaining samples is greatfully acknowledged.

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Table I. - The Reduction in COD of Synthetic Artificial Sewage by *B. circulans* under Static Conditions at 55°C.

Sample	COD	Reduction in COD
	mg/l	%
Initial	1855	0.0
2 day	1742	6.1
3 day	1510	18.6

Table II. - The Effect of Shaking on the Reduction in COD of Synthetic Artificial Sewage at 55°C by *B. circulans*.

Sample	COD	Reduction in COD
	mg/l	%
0	3710	0.0
19 hr-shake	2904	21.8
19 hr-static	3716	0.0
48 hr-static	3648	1.9

Table III. - The Effect of Aeration on the Oxidation of Domestic Sewage at 55°C by B. circulans.

Sample	COD	Reduction in COD
	mg/l	%
0	321	0.0
24 hr-static	242	24.7
24 hr-shake	224	30.2
24 hr-aerated	190	40.8

Table IV. - The Oxidation of Concentrated Domestic Sewage by B. circulans at 55°C under Aerated Conditions.

Sample	COD	Reduction in COD
	mg/l	%
0 hrs	686.6	0.0
22 hrs	408.7	40.47
51 hrs	323.4	42.90
68 hrs	299.6	56.37

Table V. - The Effect of 41 Hours Aeration at 55°C on the Reduction in COD of Sterile Domestic Sewage.

Sample	COD	pH	ml lost by
	mg/l		
initial	184.4	7.0	0
final	197.0	8.5	35

Table VI. - The Oxidation of Domestic Sewage by B. circulans under Aerated Conditions at 55°C.

Sample hrs.	COD mg/l	Reduction in COD %
0 hr-whole	174	0
0 hr-supernatant after centrifugation	132	0
18 hr-whole	148	15
18 hr-supernatant after centrifugation	70	47
43 hr-whole	108	38
43 hr-supernatant after centrifugation	86	35

2. THE DECOMPOSITION OF SEWAGE BY THERMOPHILIC MICROORGANISMS*

II. Manometric Studies

by Lewis R. Brown, David W. Cook and Robert G. Tischer

Mississippi State University, State College, Mississippi

In a preceding publication (Tischer, Brown and Cook, 1962) it was reported that the thermophilic bacterium, Bacillus circulans, would affect a reasonable reduction in the Chemical Oxygen Demand of both synthetic and normal domestic sewage. Of particular interest was the fact that this organism caused a bio-precipitation reaction in domestic sewage. Further studies conducted with this organism using manometric techniques revealed another interesting phenomenon-namely an apparently absolute requirement for carbon dioxide.

This paper will report on the results of these manometric experiments.

PROCEDURES

The dichromate reflux method was employed for determining the Chemical Oxygen Demand (COD) and the Alsterberg (azide) modification of the Winkler Method was employed in determining the Biochemical Oxygen Demand (BOD). Both procedures were conducted in accordance with the methods described in Standard Methods for the Examination of Water, Sewage and Industrial Wastes (1955).

The pH determinations were made using a Beckman Zeromatic pH meter.

Manometric studies were conducted using a Warburg Apparatus. The culture vessels were 125 ml. Warburg flasks containing 2.5 ml of substrate and cells. The center wells contained either 0.5 ml of 10% KOH

* This investigation was supported in part by a Public Health Service Research Grant No. WP-193(C1) from the Division of Water Supply and Pollution Control, National Institutes of Health, Public Health Service.

or 0.5 ml of distilled water. The temperature of incubation was 55°C. Normally 100 ml of the chosen substrate was inoculated with approximately 1 ml of a liquid culture of the test organism and the inoculated medium dispensed into the Warburg vessels.

The domestic sewage was obtained from the Starkville, Mississippi sewage system after preliminary screening. It was sterilized by autoclaving at 121°C for 15 minutes.

Concentrated sewage was prepared by boiling raw sewage under a vacuum until the volume had been reduced by approximately 75%.

Filtered sewage was prepared by filtering normal domestic sewage through a sintered glass filter (ultra fine).

RESULTS

The oxidation of domestic sewage by B. circulans. One hundred ml. amounts of normal domestic sewage, filtered domestic sewage and concentrated domestic sewage were each inoculated with 1 ml of a nutrient broth culture of B. circulans. Twenty one and a half ml amounts of each inoculated substrate were dispensed into each of 4 Warburg flasks. Two flasks of each set contained water in the center well and two flasks contained KOH in the center well. The initial pH of all substrates was 7.0, the temperature of incubation was 55°C and the duration of the experiment was 67 1/2 hours.

Very little gas consumption (approximately 400 µl) was observed in the incubated sample.

Slightly better gas consumption (540 µl with KOH in the center well and 584 µl with H_2O in the center well) was observed in the flasks containing the normal domestic sewage. Visual observation of the incubated samples revealed that the flasks containing KOH in the center well had a brownish precipitate on the sides of the flask and an essentially clear

liquid thus domonstrating the bio-precipitation phenomenon. On the other hand, the flasks containing water in the center well showed no visible precipitate but were slightly turbid.

The data obtained using the concentrated sewage are given in Table I. The bio-precipitation phenomenon was even more strikingly shown using the concentrated sewage for a heavy granular precipitate was present in the flasks containing KOH in the center well and the liquid was only slightly turbid. In the flasks containing H_2O in the center well only a trace of precipitate was present but the liquid was turbid.

In this experiment the inoculated concentrated sewage had a COD of 780.2 mg/l. From the data given in Table I it can be calculated that the reduction in COD was 28.9%. Analysis of the contents of the flasks after incubation showed a residual COD of the whole sample of 590 mg/l COD thus indicating a reduction in COD of 27.7% by chemical analysis.

This experiment was repeated using another batch of concentrated sewage (763.3 mg/l COD). The results in terms of mg/l of oxygen consumed are presented in Figure 1. Two of the flasks were sacrificed after 60 hours of incubation and the contents pooled and analyzed. The COD of these flasks was 495.6 mg/l thus revealing a reduction in COD of 267.7 mg/l which correlated well with the oxygen consumed (265.7 mg/l) by the culture after 60 hours of incubation.

The final pH values of the cultures in concentrated sewage after incubation were always high (between 9.5 and 10.2) when KOH was present in the center well. The flasks containing H_2O in the center well showed a final pH of approximately 7.5.

The oxidation of other substrates by B. circulans.

The preceding experiments seemed to indicate that the presence of CO_2 in the atmosphere above the culture influenced the metabolism of B.

circulans.

It was therefore decided to test the ability of the organism to use various proteinaceous materials in the presence of CO_2(H_2O in the center well) and in the absence of CO_2(KOH in the center well).

Each substrate was prepared in a concentration of 0.5% with 0.3% beef extract added. The media were all adjusted to pH 7.0 and sterilized. The proteins employed were Tryptone, Thiotone, Casein Hydrolysate, and Proteose Peptone No. 3. Synthetic sewage was also employed as a substrate. Each substrate was inoculated with B. circulans and dispensed in the Warburg flasks containing either KOH or H_2O in the center well. The temperature of incubation was 55°C. The results of this experiment are shown in Figures 2-6. As can be seen in Figure 2, a reasonable amount of gas was consumed using synthetic sewage as the substrate and H_2O in the center well. Surprisingly enough, however, no gas was consumed using this substrate when KOH was present in the center well. When Proteose Peptone #3 was employed as the substrate, essentially the same results were obtained (see Figure 3). Considerable gas consumption was observed when Thiotone, Tryptone and Casein Hydrolysate were used as substrates. Furthermore, greater gas consumption occurred in the flasks containing KOH in the center well than occurred in the flasks containing H_2O in the center well.

After incubation, all replicate flasks were pooled and the contents analyzed for COD. The results of these analyses as well as the COD and BOD of the original samples are given in Table II.

The oxidation of synthetic sewage by Bacillus stearothermophilus.

Bacillus stearothermophilus was tested for its ability to grow in the synthetic sewage under stationary conditions at several substrate concentrations. It was found that this organism would grow at a substrate

concentration of 2000 mg/l COD or 4000 mg/l COD but not in a concentration of 400 mg/l COD.

Synthetic sewage in the above concentrations was dispensed in 20 ml amounts in Warburg flasks and inoculated with 3.2×10^4 cells and spores (ratio of cells to spores was approximately 1:2) suspended in 1.5 ml of physiological saline solution. Other conditions of the experiment were initial pH, 7.1, temperature of incubation, 55°C and duration of 68 hours. It was found that essentially no gas was consumed by the cultures when the substrate concentration was either 400 or 2000 mg/l COD. With the substrate concentration at 4000 mg/l COD essentially no gas uptake was observed when KOH was present in the center well, but, good gas uptake (1073.4 µl) occurred in the flasks containing water in the center wells.

DISCUSSION

Very little gas consumption was observed when filtered domestic sewage was inoculated with a small number of *B. circulans* cells. This was probably due to a lack of available nutrients. Somewhat better gas consumption was observed when normal domestic sewage was employed as the substrate. It is interesting to observe that slightly better gas consumption occurred in the flasks containing H_2O in the center well. This was rather unusual since the pressure changes observed in the flasks containing H_2O in the center well are an expression of total gas consumed minus the total gas produced. Therefore, the quantity of oxygen consumed in these flasks may have been considerably more than that which was consumed in the flasks containing KOH in the center well. This seems to indicate that the metabolism of this organism is affected by the presence of CO_2.

Another point of interest is the fact that the bio-precipitation phenomenon observed earlier (Tischer, Brown and Cook, 1962) was demonstrated only in the flasks containing KOH in the center well when normal domestic sewage was employed as the substrate. This bio-precipitation

reaction was even more strikingly shown when the concentrated sewage was used as the substrate in the absence of CO_2 i. e., the flasks contained KOH in the center well. The flasks containing H_2O in the center well exhibited only a slight bio-precipitation reaction. This observation further demonstrates an apparent difference in the metabolism of this organism in the presence of CO_2. What part of this difference may be due to pH alone is not known and it may well be that this is the major factor. Obviously more information is necessary before this situation can be rationalized.

A study of the action of B. circulans on a variety of different proteinaceous substrates revealed an unexpected result; namely, that this organism was unable to germinate and/or initiate growth in several substrates in the absence of CO_2. The organism was obviously capable of growing in these substrates as was shown by the fact that gas consumption occurred in the flasks containing water in the center well. The fact that growth and gas consumption occurred in some substrates would suggest that a CO_2-by-passing mechanism was available to the organism in these substrates.

There is a tremendous amount of literature available on the subject of spore germination but only brief mention is made of a requirement of CO_2 for germination (Wynne and Foster, 1948). These workers were able to show a CO_2 requirement for the germination of Clostridium botulinum spores in synthetic medium but not in a complex medium. They were unable to retard the germination of four species of aerobic sporeformers in nutrient broth by using a CO_2-free atmosphere. No attempt was made to confirm these results with synthetic media.

Apparently for the first time, an absolute requirement for CO_2 has been observed either for spore germination and/or the initiation of growth by an aerobic sporeforming bacillus, B. circulans. Further in-

vestigations into this subject revealed that an absolute requirement for CO_2 could be demonstrated for the thermophile Bacillus stearothermophilus. For this experiment synthetic sewage was employed as the substrate at three different substrate concentrations. It was shown that this organism would grow in the medium when incubated under static conditions, but would not grow under the shake conditions imposed on it by the manometric procedures.

Furthermore, the organism did not grow at a concentration of 4000 mg/l COD when KOH was present in the center well. The only flasks showing gas consumption were those containing a substrate concentration of 4000 mg/l COD and H_2O in the center well. The seemingly conflicting findings between the results of this manometric experiment and the experiment conducted under static conditions can be rationalized on the basis that this organism requires a certain quantity of dissolved CO_2 to initiate growth. In the last experiments conducted under static conditions the concentration of dissolved CO_2 could have been increased slightly by metabolic activity and would have remained so for a longer period of time before reaching equilibrium with the gaseous atmosphere above the culture liquid. Under the shake conditions imposed on the cultures in the manometric experiments an equilibrium between the dissolved CO_2 and the CO_2 contained in the atmosphere would be reached much more quickly and consequently, unless enough CO_2 was produced to maintain the dissolved CO_2 above the critical level no growth would occur. It would appear from the results that this took place when the substrate concentration was 4000 mg/l COD but did not take place when the substrate concentration was 2000 mg/l COD.

The fact that the solubility of CO_2 is reduced essentially 50% by operating at 55°C instead of 25°C might help to explain why this absolute

requirement for CO_2 was observed in this study.

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3. Wynne, E. S. and Foster, J. W., "Physiological Studies on Spore Germination, with Special Reference to Clostridium botulinum. III. Carbon Dioxide and Germination, with a note on Carbon Dioxide and Aerobic Spores."., J. Bact. 55, 311-339, (1949).

Table I. The Oxidation of Concentrated Domestic Sewage by *B. circulans*

Sample (Hours)	µl O_2 Consumed	mg/l O_2 Consumed (Calc.)
0.0	---	---
1.5	124.0	8.8
19.0	2352.4	167.0
22.5	2495.9	178.0
43.3	3109.8	220.8
45.6	3209.3	227.9
67.5	3352.8	238.0

Table II. The Oxidation of Various Substrates by *B. circulans*

Substrate	Contents of Center Well	COD (mg/l)	% Reduction in COD	BOD 5 day 20°C (mg/l)	mg/l O_2 Consumed (24 H)	% Reduction in BOD
Thiotone (original)	---	7560	---	3000	---	---
	KOH	5746	24.0	---	1279	42.6
	H_2O	5814	23.1	---	---	---
Casein Hydrolysate (original)	---	7560	---	3785	---	---
	KOH	5916	21.7	---	1266	33.4
	H_2O	6120	19.1	---	---	---
Proteose Peptone #3 (original)	---	7080	---	2970	---	---
	KOH	---	---	---	---	---
	H_2O	5440	23.2	---	---	---
Synthetic Sewage (original)	---	7480	---	4035	---	---
	KOH	---	---	---	---	---
	H_2O	5272	25.5	---	---	---
Tryptone (orginal)	---	7836	---	2752	---	---
	KOH	5712	27.1	---	1260	45.7
	H_2O	6018	23.2	---	---	---

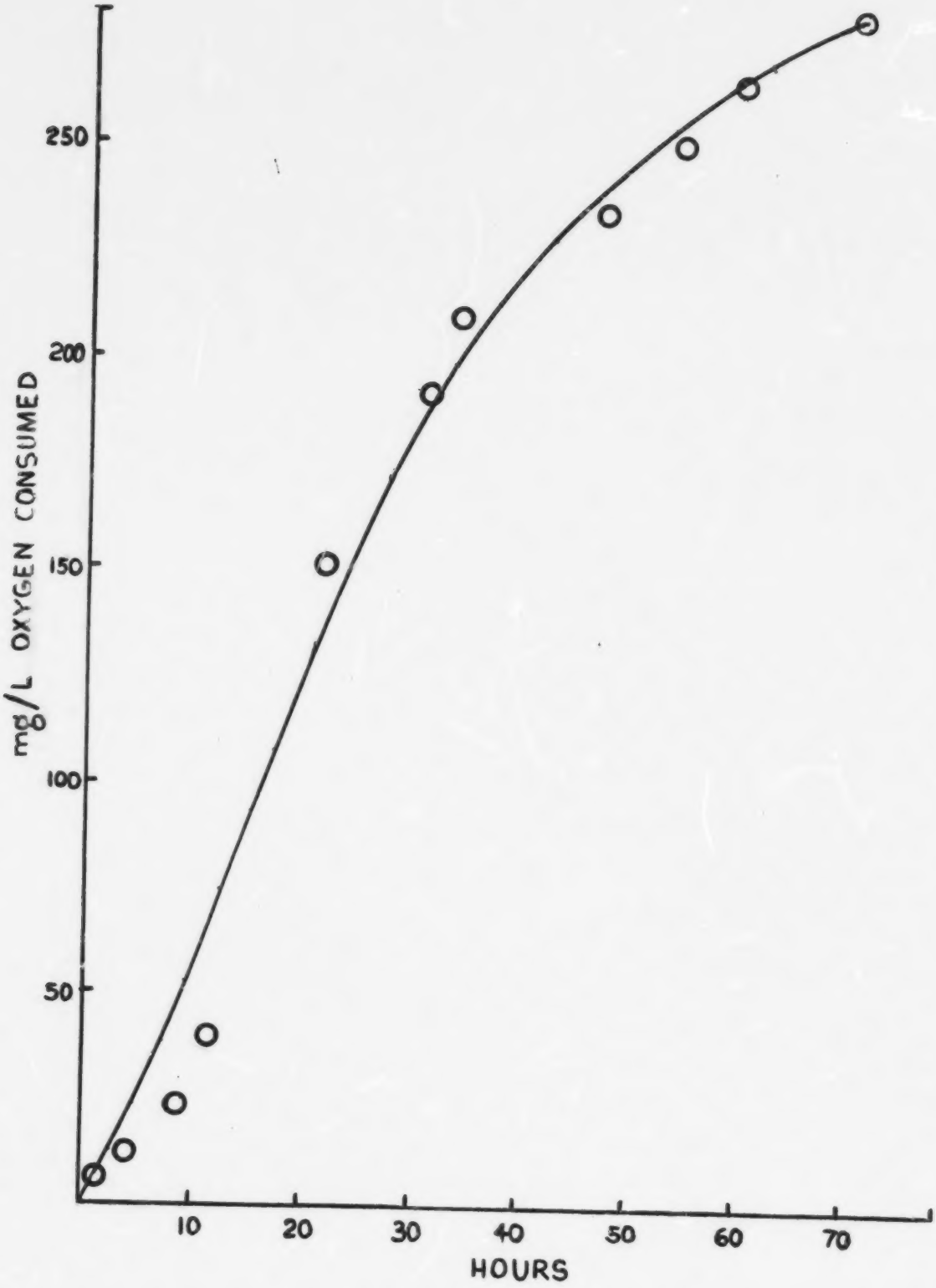

Figure 1. The Oxidation of Concentrated Domestic Sewage by B. circulans

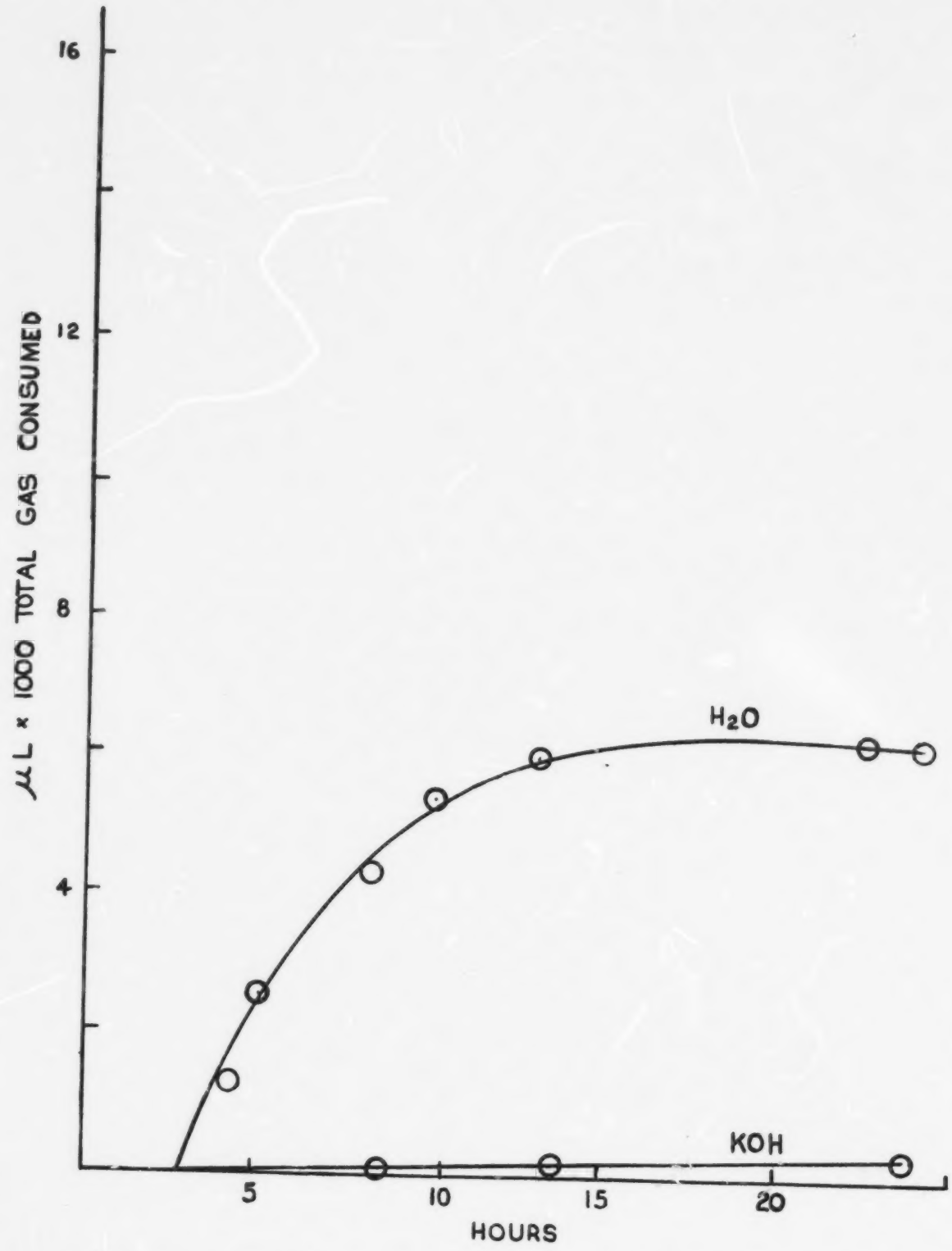

Figure 2. The Oxidation of Synthetic Sewage by B. circulans

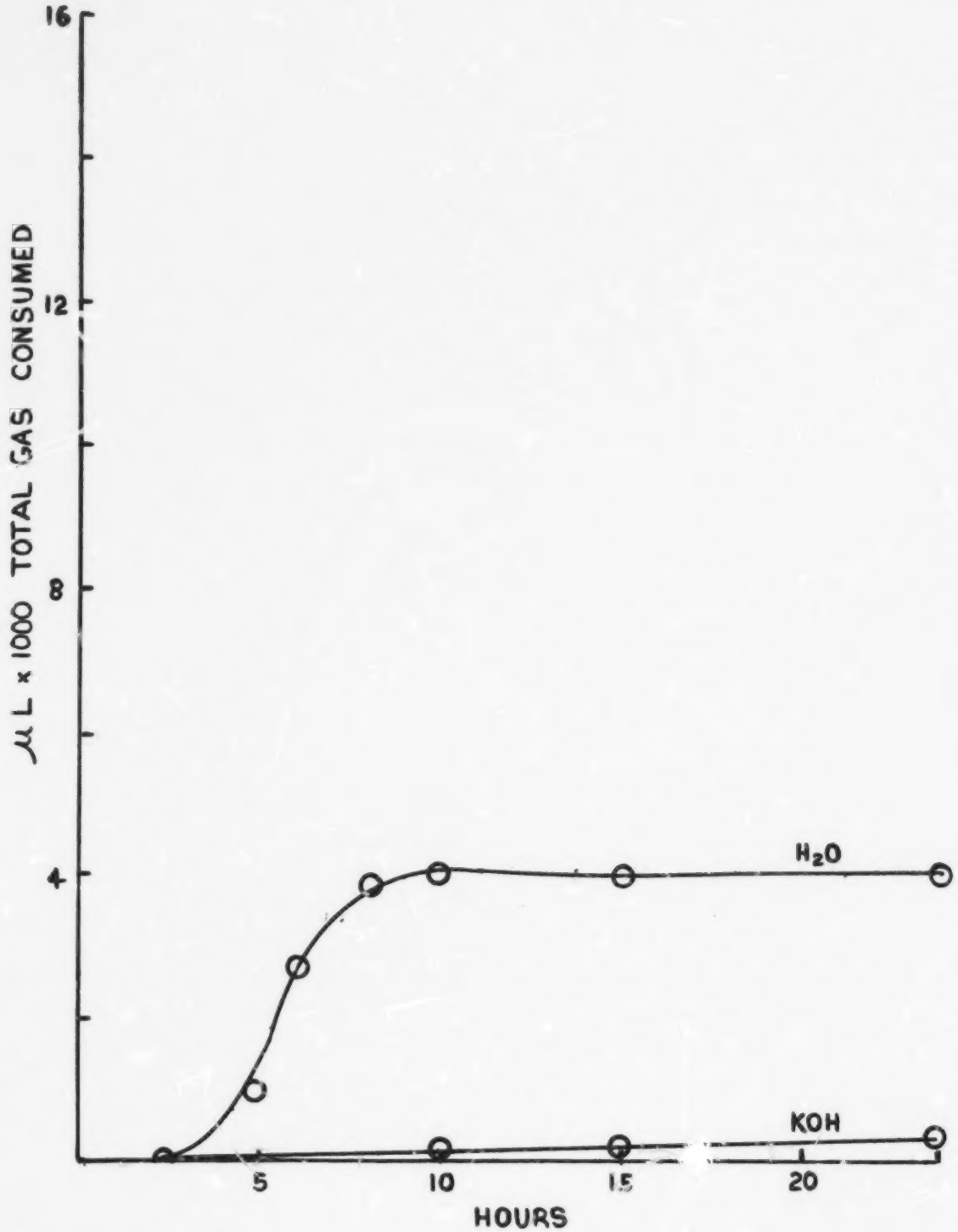

Figure 3. The Oxidation of Proteose Peptone No. 3 by B. circulans

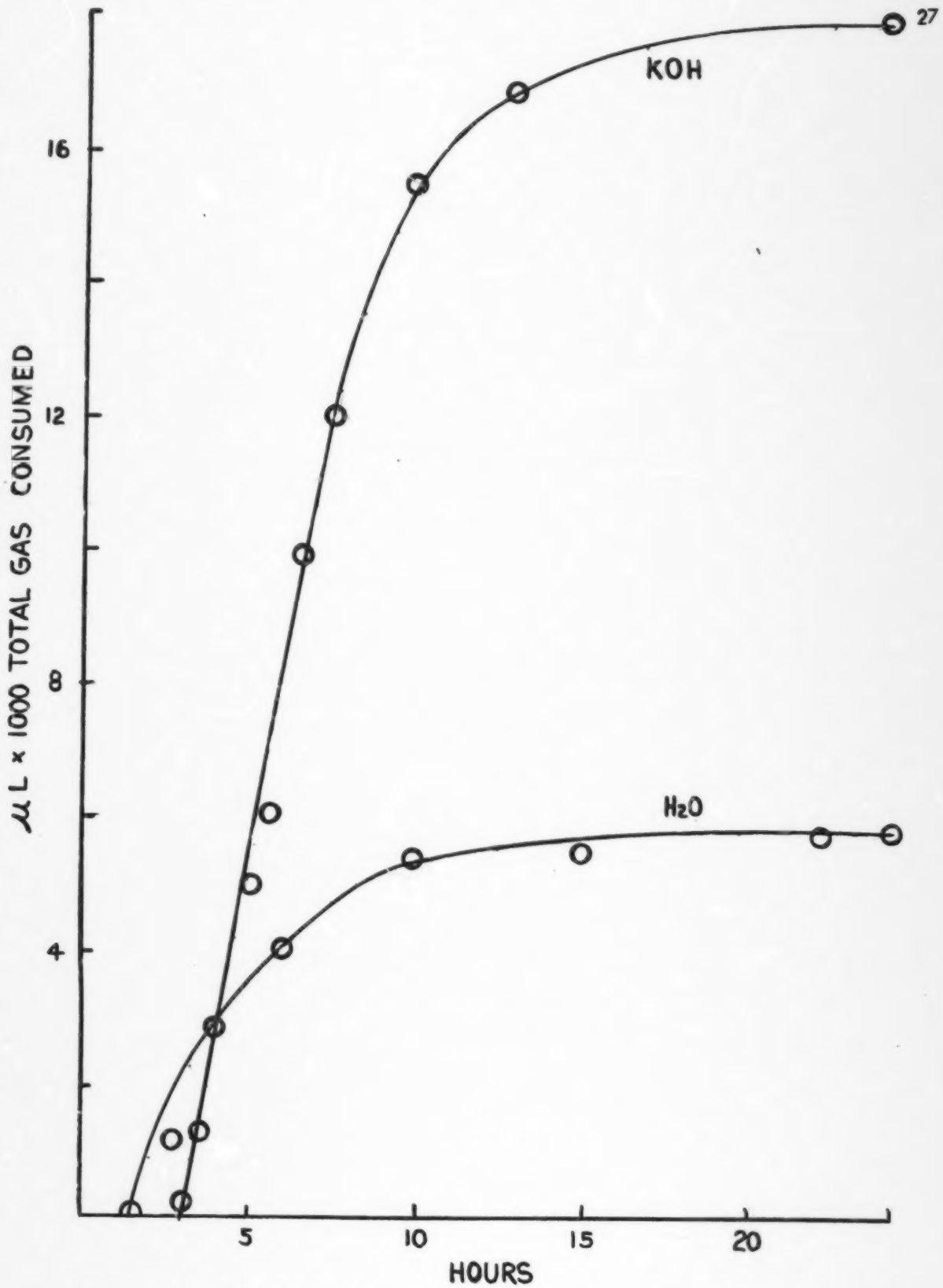

Figure 4. The Oxidation of Thiotone by B. circulans

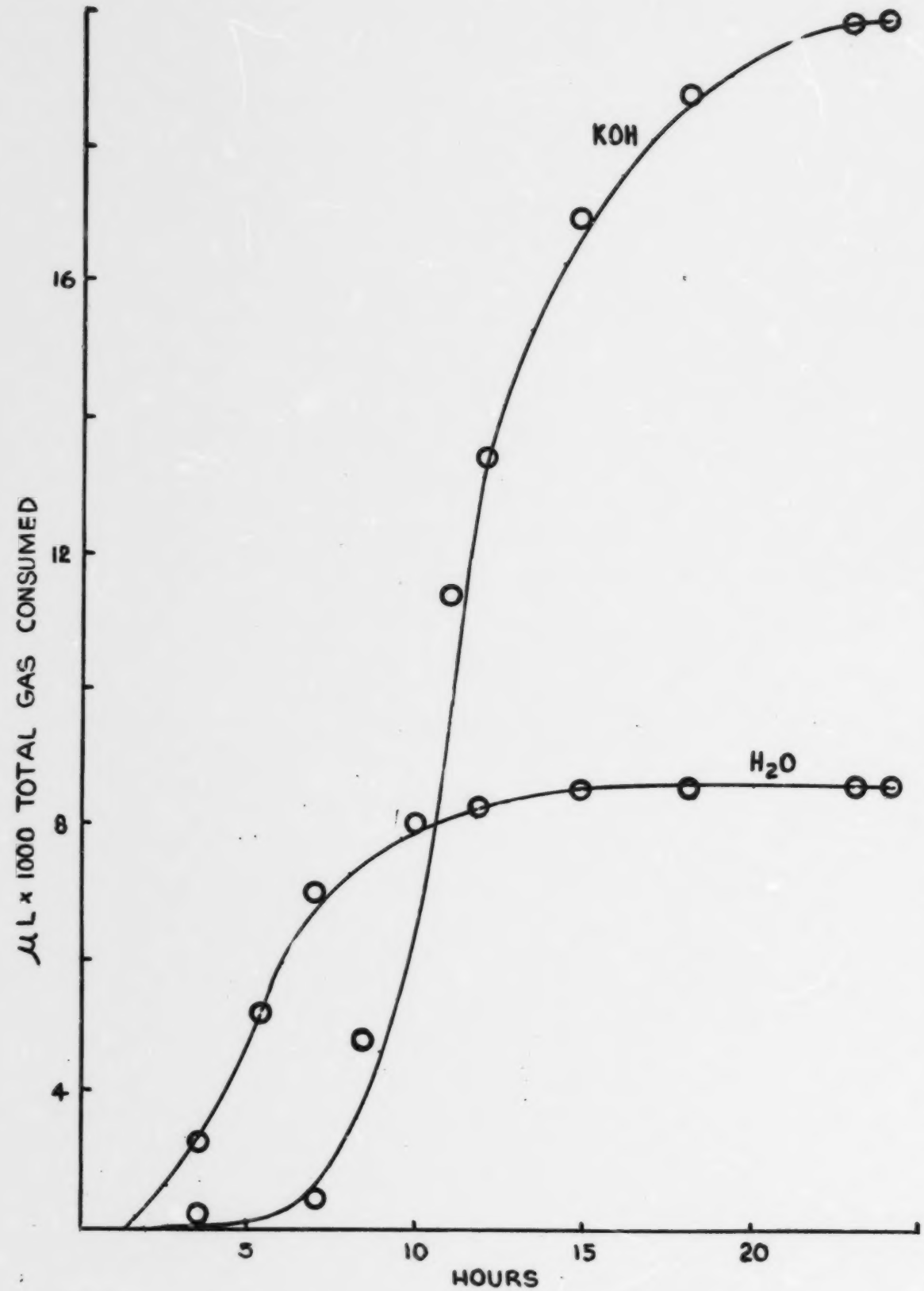

Figure 5. The Oxidation of Tryptone by B. circulans

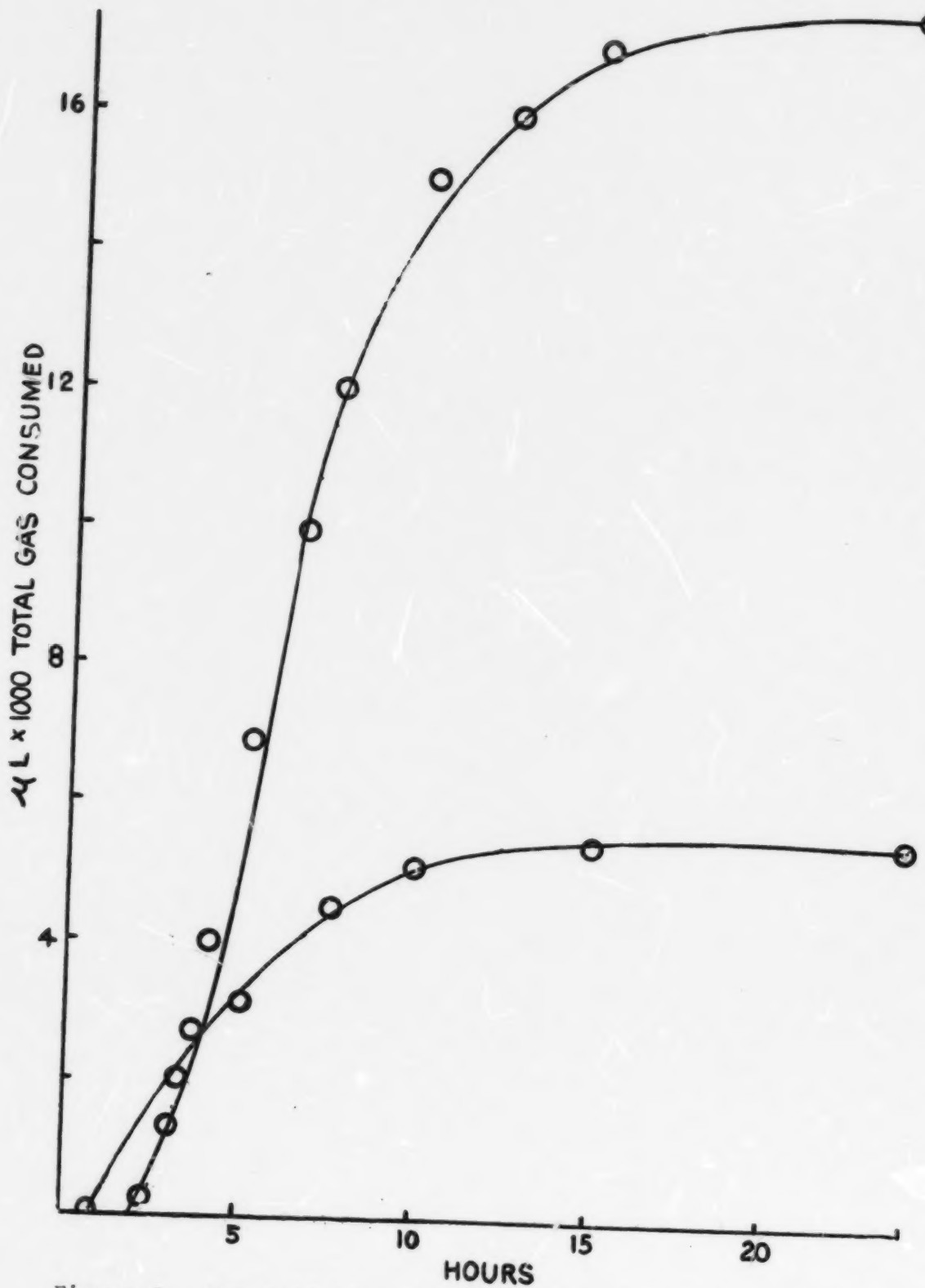

Figure 6. The Oxidation of Casein Hydrolysate by B. circulans

3. THE EFFECT OF INORGANIC IONS ON THE STABILIZATION OF DOMESTIC SEWAGE BY A THERMOPHILIC BACTERIUM*

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It has been reported that the thermophilic bacterium Bacillus circulans affects a reasonable reduction in the oxygen demand of both synthetic and normal domestic sewage (Tischer, Brown and Cook, 1962). In conducting pure culture studies using domestic sewage as the substrate, differences in the reduction of the oxygen demand were encountered. Obviously, variations in the organic constituents of the sewage could, in itself, cause these differences. Not to be overlooked, however, is the fact that the inorganic ions in sewage vary also. The question then arises as to whether variations in inorganic ions in sewage cause any significant variations in the oxidation of the sewage by B. circulans. From a practical standpoint it is of considerable interest to know whether the addition of inorganic ions to sewage would result in an increased reduction of the oxygen demand by B. circulans.

The effect of various inorganic ions on the growth of B. circulans in synthetic sewage.

One of the questions to be answered is whether the growth of B. cir- would be adversely affected by variations in some of the inorganic constituents of normal domestic sewage. The substrate of choice for these initial studies was a synthetic sewage since the composition was constant. Furthermore, the organism grows well in it and the growth is dispersed

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growth without the formation of a pellicle and without clumping for the first several days. Therefore, it was decided to employ nephelometric techniques to test the effect of various ions on the growth of B. circulans in this substrate.

The synthetic sewage employed in these experiments was Bacto-leucine assay medium, modified by the addition of leucine, sodium lauryl sulfate and stearic acid (Tischer, Brown and Cook, 1962).

Several concentrations of each inorganic compound were added to 50 ml portions of the synthetic sewage contained in 6 oz. prescription bottles. The inoculum consisted of 0.1 ml of an aqueous suspension of young B. circulans cells harvested from agar slants. Incubation was at 55°C for 17 hours under stationary conditions. The amount of growth was measured nephelometrically using a Model 9 Coleman Nepho-colorimeter. Controls, consisting of the inoculated synthetic sewage without any additions showed a nephelometer reading of 74 ± 16 nephelos units.

According to Babbitt and Baumann (1958) the concentration of nitrate in sewage varies from 0.1 mg/l to 0.4 mg/l. To test the effect of nitrate and other anions on the growth of B. circulans both the sodium and the potassium salt were employed. The nitrate concentration in the synthetic sewage was varied from 0.1 mg/l to 0.5 mg/l. All of the cultures showed nephelometer readings within the range stated above for the controls and therefore it was concluded that nitrate had no significant effect on the growth of B. circulans in the synthetic sewage.

The nitrites in sewage vary from 0 mg/l to 0.1 mg/l (Babbitt and Baumann, 1958). Cultures grown in the synthetic sewage containing from 0.05 mg/l to 0.15 mg/l nitrite showed nephelometer readings within the range for the controls and thus had no significant effect on the growth of B. circulans.

In a similar fashion it was found that the addition of chloride

ions from 15 mg/l to 225 mg/l and phosphate ions from 34.4 mg/l to 103.2 mg/l had no significant effect on the growth of the organism.

Iron in the form of ferric chloride and ferrous sulfate added to the synthetic sewage in concentrations ranging from 1 mg/l to 7 mg/l likewise failed to either inhibit or enhance the growth.

The effect of inorganic salts on the reduction in the BOD of sterile domestic sewage.

In making BOD determinations, mineral salts solutions are added to the dilution water employed to assure a sufficiency of these important inorganic compounds. It seemed of interest then to determine whether or not these same mineral salts solutions would enhance the stabilization of domestic sewage by B. circulans . The salt solutions were made up in accordance with the formulas given in Standard Methods for the Examination of Water and Wastewater, (1960), and consisted of (1) phosphate buffer solution containing ammonium chloride (2) magnesium sulfate solution (3) calciumchloride solution and (4) ferric chloride solution. The culture vessels employed were two liter Fernbach flasks containing 1 liter of sterile domestic sewage. The BOD salts solutions were added to half of the flasks in the same concentrations as normally employed in the BOD dilution water. The flasks were inoculated with B. circulans and incubated on a reciprocating shaker for 24 hours. The contents of the flasks were analyzed for their BOD content before and after incubation. The BOD analyses were conducted in accordance with Standard Methods for the Examination of Water and Wastewater, (1960). Representative data for these experiments are shown in Table I. As can be seen the BOD salts solutions did not stimulate nor inhibit the oxidation of the sewage by B. circulans. Differences in the per cent reductions were in all probability due to differences in the sewage itself. It should be pointed out that the sewage used in these experiments were grab samples and not composite samples, and had not been subjected to preliminary sedimentation.

The effect of inorganic salts on the oxidation of concentrated domestic sewage.

Since the BOD salts solutions had no effect on the oxidation of sterile domestic sewage, it was decided to determine their effect upon the oxidation of concentrated domestic sewage, since the normal inorganic constituents would be concentrated approximately 4-fold. The concentrated sewage was prepared by boiling raw sewage under vacuum until the volume had been reduced approximately 75%. After concentration, the sewage was filtered through Whatman No. 2 filter paper and autoclaved at 121°C for 15 minutes. The culture vessels for these experiments were 125 ml Warburg flasks containing 21.5 ml of inoculated concentrated sewage with and without the BOD salts added. In this case the inoculum was a small number of young B. circulans cells. Incubation was at 55°C. Half of the flasks contained water in the center well while the other half contained KOH in the center well. The results, as shown in Table II, indicate that the BOD salts inhibited the oxidation of the concentrated sewage when KOH was present in the center well. It should be pointed out, however, that after extended incubation, the oxygen consumption for both substrates was essentially the same, and thus the effect of the added salts was primarily to extend the lag phase of the organism. It is interesting to note that the pressure change in the flasks containing water in the center well were identical for all practical purposes.

Since ethylenediaminetetraacetic acid (EDTA) is a chelating agent, it was decided to test the effect of EDTA and the BOD salts on the oxidation of another batch of concentrated sewage. The conditions of this experiment were same as above with the exception of the time of incubation (20 hours in this experiment). The results are shown in Table III. With this batch of concentrated sewage the added salts inhibited the oxidation

slightly while the EDTA alone or in combination with the salts increased the oxygen consumption slightly.

Another experiment was conducted using the same batch of concentrated sewage diluted 1:1 with distilled water. As may be observed in Table IV, the added salts had essentially no effect.

The effect of inorganic salts on the germination and/or initiation of growth of B. circulans spores.

The preceding experiments have shown that added inorganic ions had little or no effect upon the growth of the test organism nor on the oxidation of either synthetic or normal domestic sewage by actively growing cultures of B. circulans. Brown, Cook and Tischer (1962) reported that spores of B. circulans had an absolute requirement for carbon dioxide for germination and/or initiation of growth in synthetic sewage. It had also been found that this requirement could be overcome in some complex media containing certain proteinaceous substances. It seemed of interest then to determine the effect of inorganic salts on the germination and/or initiation of growth of spore suspensions of B. circulans in sewage. Using spore inocula and manometric techniques it was found that the addition of the BOD salt solutions did not circumvent the CO_2 requirement in either sterile domestic sewage or concentrated sewage.

Another series of experiments was conducted using synthetic sewage in Warburg flasks with and without added calcium carbonate (300 mg carbonate per liter). These were inoculated with a spore suspension of B. circulans and incubated in the presence of CO_2 (H_2O in the center well) and in the absence of CO_2 (KOH in the center well). The results of this experiment are shown in Figure 1. As can be seen the calcium carbonate overcame the CO_2 requirement in this substrate.

The question then arises as to whether these results were due to the presence of the carbonate or the calcium. The experiment was repeated using sodium carbonate and bicarbonate as well as calcium oxide and phosphate (either 300 mg/l Ca or CO_3). Figure 2 shows that all of the flasks decreased in pressure when incubated in the presence of CO_2. Figure 3 shows that the flasks containing the sodium carbonate and bicarbonate consumed oxygen in the absence of CO_2 whereas the flasks containing the calcium oxide and phosphate had essentially no oxygen consumption. This would indicate that it was the carbonate ion rather than the calcium ion that overcame the CO_2 requirement in this substrate.

The results obtained in this investigation indicate that the addition of various inorganic ions including nitrate, nitrite, chloride, phosphate, sodium, potassium and iron had no effect of the growth of actively growing cultures of B. circulans in synthetic sewage. It would, therefore, seem unlikely that any of these ions, per se, would have any adverse effect on the growth of B. circulans in domestic sewage in the concentrations normally found in sewage. It was also found that the addition of the BOD salts solutions to normal domestic sewage failed to enhance the BOD reduction by B. circulans The addition of these salts to concentrated sewage extended the lag phase of this organism.

In the experiments using spores of B. circulans it was found that carbonate circumvented the requirement for carbon dioxide for germination and/or initiation of growth.

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3. Brown, L. R., D. W. Cook and R. G. Tischer, 1962. "The Decomposition of Sewage by Thermophilic Microorganisms. II. Manometric Studies.", J. Water Pol. Cont. Fed. (accepted for publication).

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Table I. The effect of inorganic salts on the reduction of the BOD of sterile domestic sewage by Bacillus circulans

Sample	Initial BOD mg/l	Final BOD mg/l	BOD Reduction %
Sewage A	225.0	82.5	63.2.2
Sewage A + Salts	225.0	80.5	64.0
Sewage B	127.0	53.0	58.3
Sewage B + Salts	127.0	59.0	53.6
Sewage C	110.0	86.0	22.0
Sewage C + Salts	112.0	86.0	23.0

Table II. The effect of inorganic salts on the oxidation of concentrated domestic sewage by Bacillus circulans after 27 hours in incubation.

Measurement	Concentrated Sewage	Concentrated Sewage and Salts
Oxygen consumed, µ l	4039	2535
Oxygen consumed, mg/l	287	180
Initial BOD, mg/l	424	424
BOD reduction, %	67.7	42.5
Pressure change with H_2O in the center well, µl	2322	2327

Table II. The effect of inroganic salts and EDTA on the oxidation of concentrated domestic sewage by Bacillus circulans in 20 hours.

Measurement	Concentrated Sewage	Concentrated Sewage & Salts	Concentrated Sewage & EDTA**	Concentrated Sewage & Salts & EDTA**
Oxygen consumed, μ l	2403	2197	2451	2499
Oxygen consumed, mg/l	171	156	174	177
Initial BOD*, mg/l	270	270	270	270
BOD Reduction, %	63.3	57.8	64.4	65.6
Pressure change with H_2O in the center well, μ l	1159	1087	1168	1133

* The initial BOD was determined for the concentrated sewage without added salts or EDTA.

** The concentration of EDTA in the sewage was 0.01%

Table IV. The effect of inorganic salts on the oxidation of concentrated domestic sewage diluted 1:1 with water by *Bacillus circulans* in 20 hours.

Measurement	Concentrated sewage & water (1:1)	Concentrated sewage & water (1:1) + salts
Oxygen consumed, µl	997	1018
Oxygen consumed, mg/l	71	72
Initial BOD*, mg/l	135	135
BOD reduction, %	52.6	53.3
Pressure change with H_2O in the center well, µl	575	474

* The concentrated sewage had a BOD of 270 mg/l.

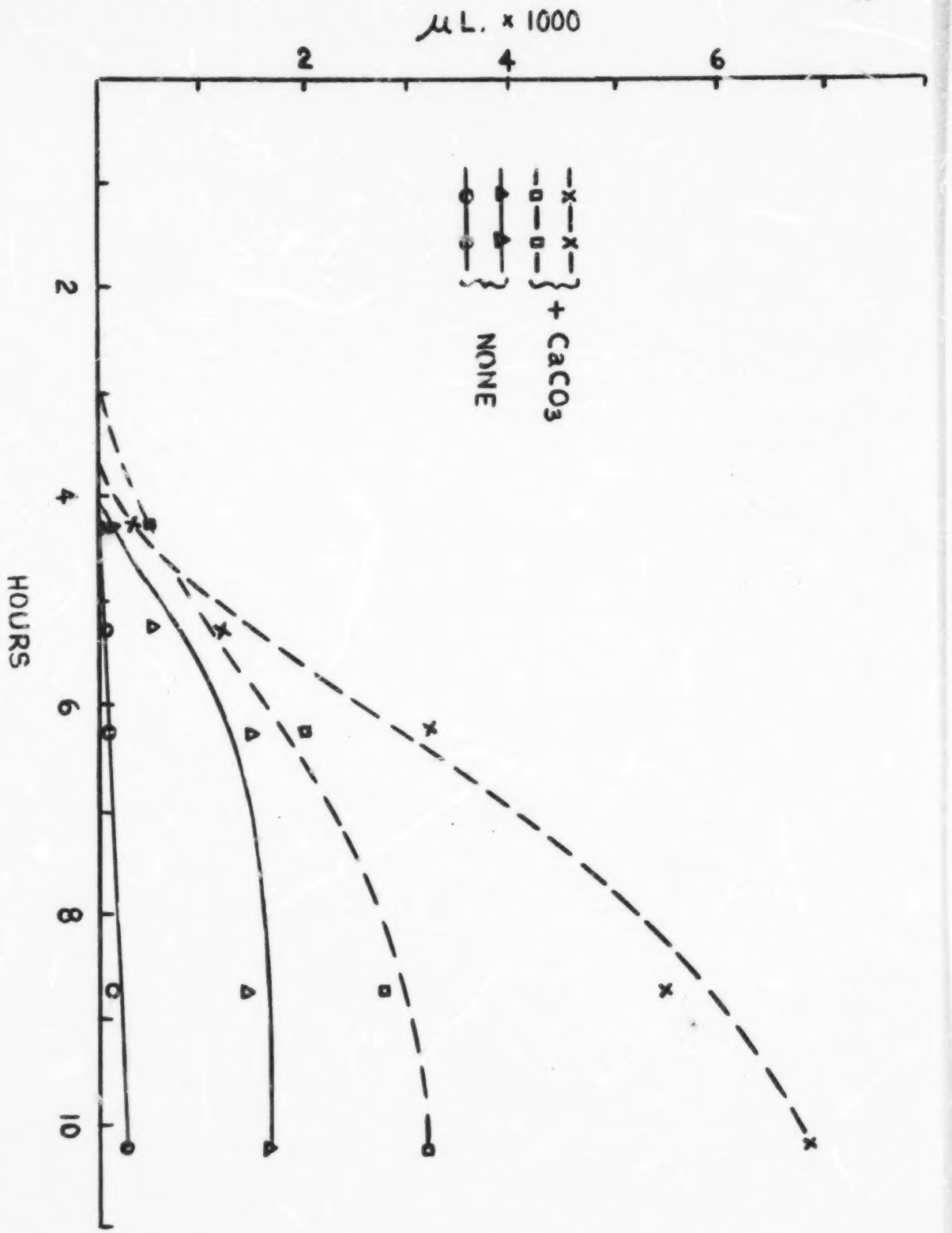

Figure 1. The effect of calcium carbonate on the oxidation of synthetic sewage inoculated with spores of B. circulans

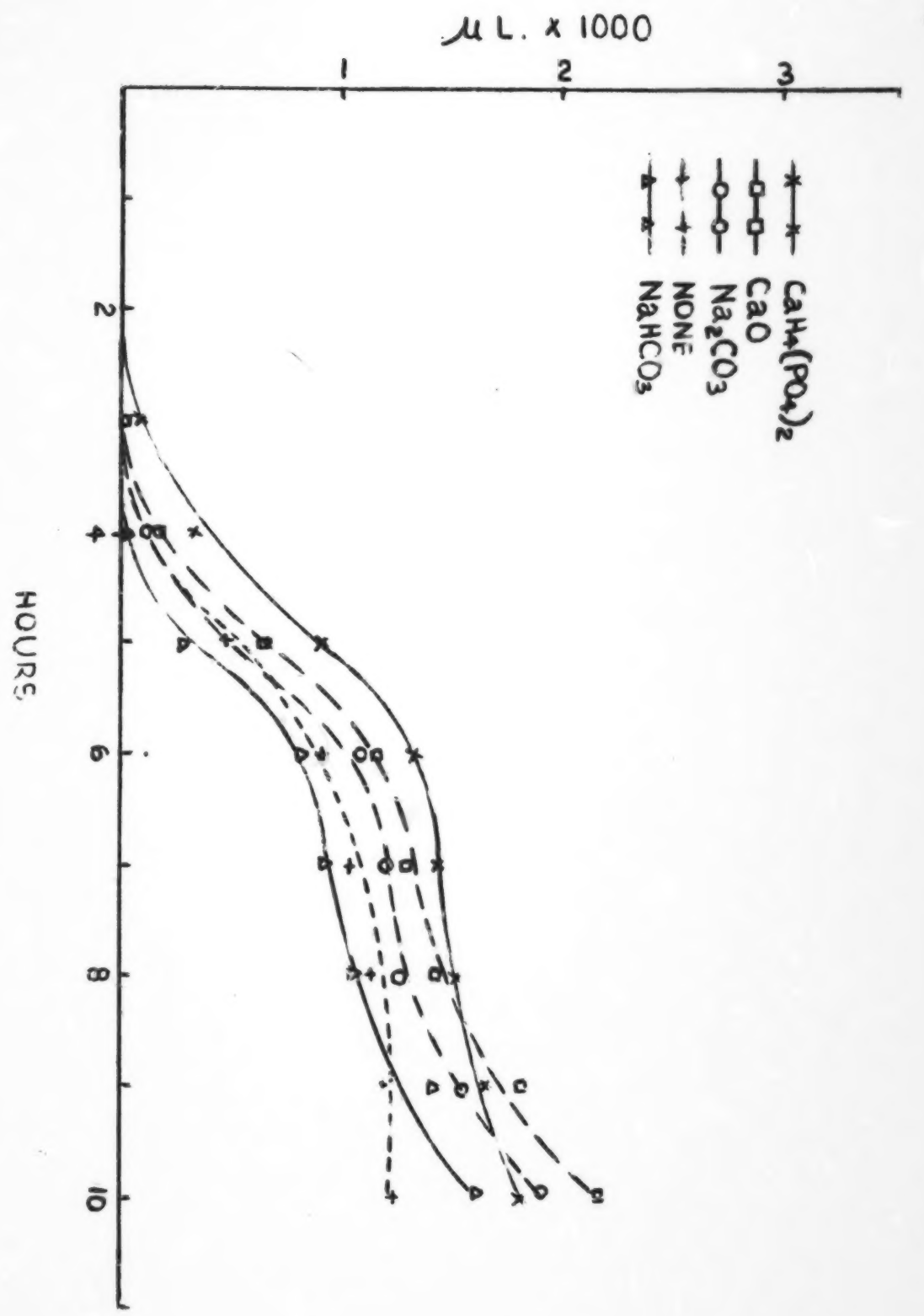

Figure 2. The effect of calcium ions and carbonate ions on the oxidation of synthetic sewage inoculated with spores of B. circulans and grown in the presence of carbon dioxide.

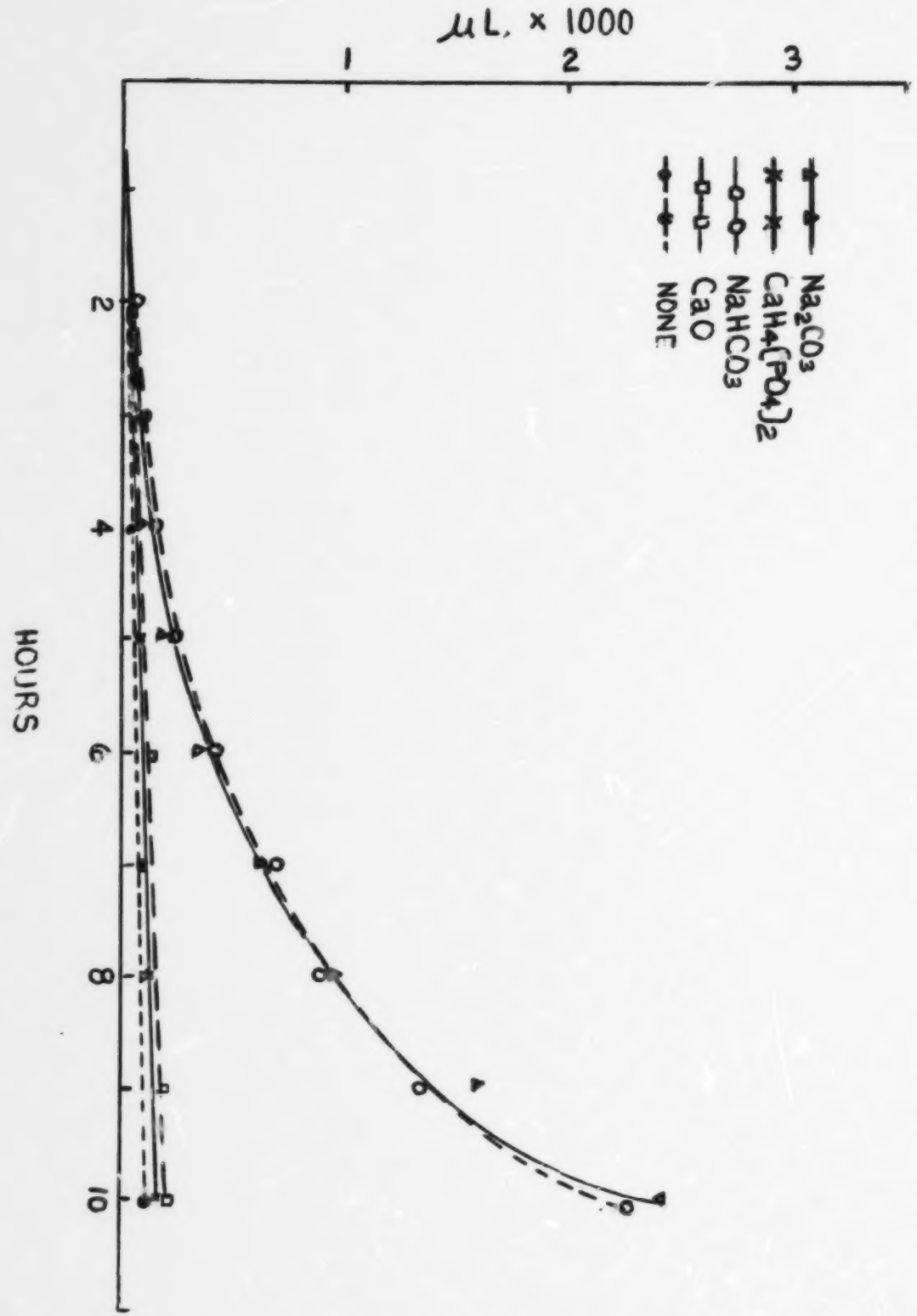

Figure 3. The effect of calcium ions and carbonate ions on the oxidation of synthetic sewage inoculated with spores of B. circulans and grown in the absence of carbon dioxide.

4. THE EFFECT OF THERMOPHILIC TEMPERATURES ON THE MICRO-ORGANISMS IN DOMESTIC SEWAGE.

INTRODUCTION

One of the factors to be considered in a thermophilic sewage treatment process is the fate of the organisms normally present in sewage. Since the temperature of operation is 55°C or above, the sewage would in essence be pasteurized and thus postchlorination of the effluent may be precluded. This section of the report will deal with this aspect of the system.

METHODS

The most probable number (MPN) determinations were carried out using standard lactose broth. The Confirmed test was carried out using Eosin Methylene Blue Agar. Both of the above tests as well as the completed test were carried out in accordance with Standard Methods for the Examination of Water and Wastewater (1960).

Plate counts were conducted using tryptone glucose yeast extract agar and the conventional pour plate procedure.

RESULTS

One liter of normal domestic sewage contained in a 2 liter Fernbach flask was heated to 55°C in a water bath in approximately 2 minutes. The culture was then transferred to a round bottomed flask and aerated for 24 hours. Samples were withdrawn after 1, 2 and 24 hours of incubation. The original sewage was analyzed before heating. The results of this experiment are given in Table I. These results seemed to indicate that coliform organisms survived

this treatment and, in fact, after an initial drop in numbers, increased in number.

This experiment was repeated under shake conditions using 1.5 liters of domestic sewage contained in 2 liter Fernbach flasks. Incubation was at 55°C for 20 hours. The results are given in Table II. All samples showing gas in the lactose broth were streaked on Eosin Methylene Blue Agar and incubated at 35 ± 0.5°C. As was expected, all of the tubes from the original samples showing gas yielded typical coliform colonies on the EMB agar whereas none of the tubes from the final samples showing gas had typical colonies.

The typical colonies from the EMB plates were subjected to the completed test and gave a positive coliform test.

DISCUSSION

The results presented in this section are preliminary findings only. It should be pointed out, however, that these experiments have been repeated many times and have never shown the presence of coliform organisms in sewage after incubation at 55°C for 20 hours. Studies are presently under way to isolate and identify the organisms responsible for the false positive presumptive tests.

Table I. The effect of aeration at 55°C on the organisms in sewage capable of producing gas in Lactose broth.

Sample	MPN at 24 hrs	Reduction	MPN at 48 hrs	Reduction
	no/100 ml	%	no/100 ml	%
0 hr	5,400,000	--	9,200,000	--
1 hr	240	99.995	350	99.996
2 hr	3,500	99.93	54,000	99.41
24 hr	2,400	99.95	9,200	99.9

Table II. The effect of 20 hours incubation at 55°C under shake conditions on the organisms in sewage capable of producing gas in Lactose broth.

Sample	MPN at 24 hrs	Reduction	MPN at 48 hrs	Reduction
	no/100 ml	%	no/100 ml	%
Sewage A original	24,000,000	--	35,000,000	--
Sewage A Final	950	99.996	24,000	99.92
Sewage B Original	54,000,000	--	54,000,000	--
Sewage B Final	460	99.9991	35,000	99.93
Sewage C Original	24,000,000	--	24,000,000	--
Sewage C Final	490	99.998	17,000	99.93

4. LIST OF PUBLICATIONS

Tischer, R. G., Brown, L. R., and Cook, D. W., "The Decomposition of Sewage by Thermophilic Microorganisms. I. Preliminary Studies." (accepted for publication by Jour. Water Pol. Cont. Fed.)

Brown, L. R., Cook, D. W. and Tischer, R. G., "The Decomposition of Sewage by Thermophilic Microorganisms. II. Manometric Studies." (accepted for publication by Jour. Water Pol. Cont. Fed.)

Tischer, R. G., Cook, D. W. and Brown, L. R., "The Effect of Inorganic Ions on the Stabilization of Domestic Sewage by a Thermophilic Bacterium. (submitted for publication in Advan. in Industr. Microbiol.)

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6. FOREIGN TRAVEL

None.